Developmental Cell
Article

MIWI2 Is Essential for Spermatogenesis and Repression of Transposons in the Mouse Male Germline

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DOI 10.1016/j.devcel.2007.03.001

SUMMARY

Small RNAs associate with Argonaute proteins and serve as sequence-specific guides for regulation of mRNA stability, productive translation, chromatin organization, and genome structure. In animals, the Argonaute superfamily segregates into two clades. The Argonaute clade acts in RNAi and in microRNA-mediated gene regulation in partnership with 21–22 nt RNAs. The Piwi clade, and their 26–30 nt piRNA partners, have yet to be assigned definitive functions. In mice, two Piwi-family members have been demonstrated to have essential roles in spermatogenesis. Here, we examine the effects of disrupting the gene encoding the third family member, MIWI2. *Miwi2*-deficient mice display a meiotic-progression defect in early prophase of meiosis I and a marked and progressive loss of germ cells with age. These phenotypes may be linked to an inappropriate activation of transposable elements detected in *Miwi2* mutants. Our observations suggest a conserved function for Piwi-clade proteins in the control of transposons in the germline.

INTRODUCTION

Argonaute proteins lie at the heart of RISC, the RNAi effector complex, and are defined by the presence of two domains, PAZ and Piwi. Phylogenetic analysis of PAZ- and Piwi-containing proteins in animals suggests that they form two distinct clades, with several orphans. One clade is most similar to *Arabidopsis* ARGONAUTE1. Proteins of this class use siRNAs and microRNAs as sequence-specific guides for the selection of silencing targets. The second clade is more similar to *Drosophila* PIWI. Like Argonautes, Piwi proteins have been implicated in gene-silencing events, both transcriptional and posttranscriptional.

Piwi-clade proteins have been best studied in the fly, which possesses three such proteins: PIWI, AUBERGINE, and AGO3. Until recently, evidence for the involvement of Piwi proteins in gene silencing was mainly genetic. The first biochemical insight into the biological role of Piwi-family proteins was the observation that both PIWI and AUBERGINE exist in complexes with repeat-associated siRNAs (rasiRNAs) (Saito et al., 2006; Vagin et al., 2006). RasiRNAs were first described in *Drosophila* as 24–26 nt, small RNAs corresponding to repetitive elements, including transposons (Aravin et al., 2001, 2003). The interaction between Piwi proteins and rasiRNAs dovetails nicely with the observation that, in *Drosophila*, both *piwi* and *aubergine* are important for the silencing of repetitive elements.

Mutations in Piwi-family genes cause defects in germline development in multiple organisms. For example, in flies, *piwi* is necessary for self-renewing divisions of germline stem cells in both males and females (Cox et al., 1998; Lin and Spradling, 1997). Mutations in *aubergine* cause male sterility and maternal effect lethality (Schmidt et al., 1999). The male sterility is directly attributable to the failure to silence the repetitive *stellate* locus. Mutant testes also suffer from meiotic nondisjunction of sex chromosomes and autosomes (Schmidt et al., 1999). A recent study indicates that the sterility observed in female flies bearing mutations in Piwi-family proteins is also likely to result, at least in part, from the deleterious effects of transposon activation (Brennecke et al., 2007).

As is seen in other organisms, the expression of the three murine Piwi proteins, MIWI (PIWIL1), MILI (PIWIL2),

and MIWI2 (PIWIL4), is largely germline restricted (Kuramochi-Miyagawa et al., 2001; Sasaki et al., 2003). Thus far, MIWI and MILI have been characterized in some detail, with mice bearing targeted mutations in either *Miwi* (Deng and Lin, 2002) or *Mili* (Kuramochi-Miyagawa et al., 2004) being male sterile. Although both MIWI and MILI are involved in regulation of spermatogenesis, loss of either protein produces distinct defects that are thematically different from those seen upon mutation of *Drosophila piwi*. Based upon their expression patterns and the reported phenotypes of mutants lacking each protein, the most parsimonious model is that both MIWI and MILI perform roles essential for the meiotic process. So far, no mammalian Piwi protein has a demonstrated role in stem cell maintenance as proposed for *Drosophila* PIWI. This raised the possibility that any role for mammalian Piwi proteins in stem cell maintenance might reside in the third family member, MIWI2.

Despite the presence of conserved RNA-binding motifs and an expectation that mammalian Piwi proteins might be involved in RNA-induced silencing mechanisms, no interaction was described for these proteins with siRNAs or miRNAs. Recently, we and others identified small RNA binding partners for Piwi proteins in the male germline, designated as piRNAs (Piwi-Interacting RNAs) (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Watanabe et al., 2006). piRNAs show distinctive localization patterns in the genome. They are predominantly grouped into 20–90 kb genomic regions, wherein numerous small RNAs are produced from only one genomic strand. Most piRNAs match the genome at unique sites, and less than 20% match repetitive elements. piRNAs become abundant in germ cells around the pachytene stage of prophase of meiosis I, but they may be present at lower levels during earlier stages. Unlike microRNAs, individual piRNAs are not conserved. Thus, it is difficult to intuit biological function based on the sequence content of piRNA populations.

To investigate the role of MIWI2 in gametogenesis, we disrupted the gene encoding this third mouse Piwi-family member. We find that *Miwi2* mutants have two discrete defects in spermatogenesis. The first is a specific meiotic block in prophase of meiosis I that exhibits distinctive morphological features. This is followed by a progressive loss of germ cells from the seminiferous tubules. These phenotypes, and the fact that *Miwi2* is expressed both in germline and somatic compartments, highlight similarities between MIWI2 and *Drosophila* PIWI. In this regard, we find that disruption of *Miwi2* also interferes with transposon silencing in the male germline.

RESULTS

Miwi2 Mutants Have Defects in Spermatogenesis

We used an insertional mutagenesis strategy to disrupt the *Miwi2* gene (Figures 1A and 1B). The allele that we created contains a 10 kb segment of vector sequence following *Miwi2* exon 12. Downstream of the vector insertion, the genomic region encompassing exons 9–12 is duplicated. This is predicted to insert multiple in-frame stop codons and to produce a nonfunctional allele. When primers downstream of the insertion are used, quantitative RT-PCR indicates that *Miwi2* transcripts are essentially undetectable in homozygous mutant animals at 10 days postpartum (dpp), before mutants phenotypically diverge from wild-type (Figure S1; see the Supplemental Data available with this article online). This is precisely what would be expected if nonsense-mediated decay were acting on the predicted mRNA containing numerous premature stop codons. However, all of the coding capacity of *Miwi2* still exists in the mutant genome, and splicing around the insertion could conceivably produce a functional *Miwi2* transcript. Using RT-PCR primers that flank the duplicated exons, we could not detect any wild-type transcript that would be produced by such a splicing event in *Miwi2* mutant animals (Figure 1C). Therefore, we can assert with confidence that our allele produces, at the very least, a severe hypomorph and is likely a null allele.

Mice heterozygous for the *Miwi2* mutant allele grew to adulthood, were fertile, and appeared phenotypically normal. Upon intercrossing, it became obvious that male mice homozygous for a mutant allele of *Miwi2* were infertile, although they exhibited normal sexual behavior. Homozygous females, however, were fertile and had no obvious defects. Males and females of both sexes were of normal size and weight and had the expected life span.

Initial examination of testes of adult *Miwi2* mutants revealed a very obvious and severe phenotype. Although all other reproductive organs were of normal size and appearance, *Miwi2* mutant testes were substantially smaller than their wild-type or heterozygous counterparts (Figure 1D). In juveniles at 10 dpp, wild-type and mutant testes were indistinguishable both morphologically (not shown) and histologically (Figures 2A and 2B). However, cellular defects became apparent a few days later as germ cells proceeded through the first round of spermatogenesis.

Mouse spermatogenesis is a highly regular process that takes ~35 days to complete (de Rooij and Grootegoed, 1998). Spermatogonia, a very small percentage of which are stem cells, line the periphery of the seminiferous tubule and divide mitotically to maintain the stem cell population throughout the lifetime of the animal. These divisions also give rise to differentiating cells that undergo several rounds of mitotic division before entering meiosis. Meiotic cells, or spermatocytes, advance through meiotic prophase I, which can be separated into five phases. In leptotene (phase 1), duplicated chromosomes begin to condense. More extensive pairing and the formation of synaptonemal complexes occur in zygotene (phase 2) and are completed in pachytene (phase 3), when crossing over occurs. Homologs begin to separate in diplotene (phase 4), and chromosomes move apart in diakinesis (phase 5). Prophase I is followed by two meiotic divisions that eventually generate haploid products. The immediate product of meiosis is the round spermatid, which will mature and elongate until being released into the lumen of the tubule.

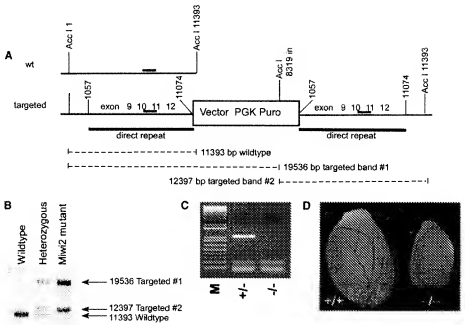

Figure 1. Generation of a Mutant *Miwi2* Allele

(A) The insertional disruption strategy for *Miwi2* is shown schematically. The insertion duplicates exons 9–12. Approximately 10 kb of vector sequence is also inserted into the gene.

(B) Wild-type, heterozygous, and homozygous mutant animals were identified by Southern blot analysis using the probe indicated by the red bar in (A). The targeted allele gives two signals, both distinct from wild-type, because the probe is within the duplicated region.

(C) RT-PCR was used to amplify wild-type *Miwi2* transcripts in testes of 14-day-old animals.

(D) Gross appearance of wild-type and mutant testes at 6 weeks of age.

At the stage when tubules of wild-type siblings contained germ cells at the zygotene and pachytene phases of meiosis I, germ cells in the mutant became noticeably atypical (Figures 2C–2F). Two abnormal nuclear morphologies were observed in mutant spermatocytes. In ~80% of abnormal spermatocytes, the nuclei were very condensed and stained intensely with hematoxylin and DAPI (Figures 2D and 2F, arrow). The remaining 20% of abnormal nuclei were extremely large and had an "exploded" morphology with apparently scattered chromatin (Figures 2D and 2F, arrowhead). The two types of abnormal nuclei appear simultaneously. Therefore, it is unlikely that the same cell transitions from one nuclear morphology to the other. Mutant spermatocytes never proceeded further into, or completed, meiosis I. Consequently, histological examination also revealed that mutant testes contained no postmeiotic cell types such as haploid spermatids or mature sperm. Instead, mutant testes degenerated with age (Figures 2G and 2H).

Meiotic Defects in *Miwi2* Mutants

To examine the apparent meiotic defect more closely, we tracked the progress of synapsis by using spermatocyte spreads (Figure 3). When spreads were prepared from mutant testes, the vast majority of spermatocytes (>95%) were in the leptotene stage (Figure 3D). At this stage, Scp3, a component of the axial element of the synaptonemal complex, becomes associated with the two sister chromatids of each homolog (Lammers et al., 1994; Moens et al., 1987). Only a few percent of mutant spermatocytes reached zygotene, when longer paired and unpaired axial elements are observed (Figures 3E and 3F). Normal pachytene spermatocytes (e.g., Figure 3C) with fully condensed, paired chromosomes were never observed in mutant animals.

Phosphorylated histone H2AX (γ-H2AX) marks the sites of Spo11-induced DNA double-strand breaks that occur during leptotene (Celeste et al., 2002; Fernandez-Capetillo et al., 2003; Hamer et al., 2003; Mahadevaiah et al., 2001). In wild-type cells, double-strand breaks were repaired normally, and most of the γ-H2AX signal disappeared as cells entered pachytene (Figure 4A, P = pachytene). In *Miwi2* mutant spermatocytes, γ-H2AX staining appeared normal during the leptotene stage (compare Figures 4A and 4B, L = leptotene). However, concomitant with the change in morphology to highly condensed nuclei, mutant spermatocytes appeared to stain more intensely for γ-H2AX (Figure 4D, AS = abnormal spermatocyte) as compared to wild-type zygotene cells (Figure 4C, Z = zytogene). The persistence and strength of the γ-H2AX staining may indicate the presence of unrepaired double-strand breaks and/or widespread

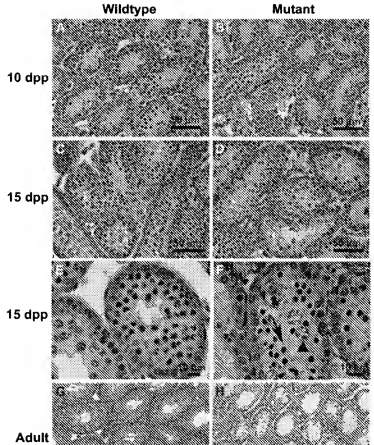

Figure 2. Histological Analysis Reveals Abnormalities in *Miwi2* Mutants

(A–H) Hematoxylin and eosin staining of (A, C, E, and G) wild-type and (B, D, F, and H) mutant testes at indicated ages. (A)–(F) depict the first wave of spermatogenesis at (A and B) 10 days post partum (dpp) and (C–F) 15 dpp. Adult testes from 3-month-old animals are depicted in (G) (wild-type) and (H) (mutant). The arrow and arrowhead indicate two types of abnormal mutant spermatocytes (see text).

asynapsis, as the cells failed to progress successfully to pachytene. Similar patterns have been observed previously, as mutants defective in synapsis or double-strand break repair fail to eliminate γ-H2AX from bulk chromatin (Barchi et al., 2005; Wang and Hoog, 2006; Xu et al., 2003).

During male meiotic prophase, the incorporation of the X and Y chromosomes into the sex or XY body correlates with their transcriptional silencing. By pachytene stage, a second wave of γ-H2AX accumulates in the sex body in association with the unsynapsed axial cores of the sex chromosomes (de Vries et al., 2005; Turner et al., 2005) (Figures 4A and 4E). When using standard histological staining, the "exploded" nuclei in *Miwi2* mutants often contained structures that look remarkably like sex bodies (Solari, 1974) (Figure 4F); however, these fail to stain with γ-H2AX despite its appearance on the scattered chromatin (Figure 4F). At this time, it is unknown whether these structures contain the sex chromosomes or whether other proteins known to populate the sex body are present. This structure may also be a nuclear organelle, such as the nucleolus, that is not normally as prominent at this stage. Nevertheless, we consistently fail to observe a γ-H2AX focus in *Miwi2* mutants that is characteristic of a successfully formed sex body.

As *Miwi2* mutant animals aged, they exhibited dramatically increased levels of apoptosis in the seminiferous tubules as compared to wild-type (Figure 5). A fluorescent TUNEL assay revealed that, while a section through a wild-type testis (Figure 5A) showed few or no apoptotic cells (green), a large fraction of tubules in the mutant had many dying cells (Figure 5B). These developmental abnormalities arose during prophase of meiosis I. Although occasional TUNEL-positive spermatocytes were present in many tubule sections, larger groups of apoptotic spermatocytes were found in epithelial stage IV, characterized by the presence of mitotic intermediate spermatogonia and early B spermatogonia (Figure 5C). The apoptosis of spermatocytes in stage IV resulted in the absence of spermatocytes in later stages, except for a few that entered apoptosis a little more slowly and disappeared in stages V–VII. While the apoptosis of virtually all spermatocytes in stage IV has been observed in many mutants defective

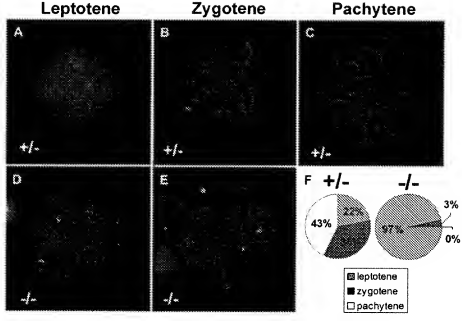

Figure 3. Mutant Spermatocytes Arrest before the Pachytene Stage of Meiosis I
(A–E) Spermatocyte spreads from (A–C) heterozygous and (D and E) mutant animals in leptotene, zygotene, and pachytene stages, as indicated. (F) Percentage of nuclei in each stage.

in meiotic genes (Barchi et al., 2005; de Rooij and de Boer, 2003), the *Miwi2* mutation elicits a unique spermatocyte behavior, as they either condense or enlarge long before they reach epithelial stage IV and apoptose (Figure 5D). In light of these results, we concluded that the seemingly more intense γ-H2AX staining of mutant spermatocytes (Figure 4D) was not due to the creation of double-strand breaks upon induction of apoptosis, as the observed tubules had not yet reached stage IV.

Miwi2 Mutants Deplete Germ Cell Lineages

As mutant animals aged, their seminiferous tubules became increasingly vacuolar (Figure 2H). Staining with germ cell nuclear antigen (GCNA), which is expressed in all germ cells, indicated that *Miwi2* mutants exhibited a marked decrease in the number of germ cells with age (Figures 6A–6D). Before the onset of meiosis, the number of germ cells was indistinguishable from that in wild-type (Figures 2A and 2B). However, with age, mutant tubules contained fewer spermatogonia and abnormal spermatocytes. Tubules lacking germ cells and containing only Sertoli cells began appearing as early as 3 months of age. As the animals aged, Sertoli-cell-only tubules increased in number and became predominant. The Sertoli cells that populate these germ cell-less tubules appeared histologically normal.

Spermatogenic failure and germ cell loss can result from defects in germ cells or in their somatic environment (Brinster, 2002). In addition to being expressed in premeiotic germ cells, *Miwi2* is expressed at significant levels in c-kit mutant testes (*W/W*^{v}) that are virtually germ cell free (Silvers, 1979) and is also detectable in the TM4 Sertoli cell line (Figure S1; M. Griswold, personal communication). Thus, we sought to determine whether the defects observed in *Miwi2* mutant testes reflect a cell-autonomous defect in the germ cells themselves or whether MIWI2 plays a critical role in somatic support cells.

To address this question, we transplanted wild-type germ cells into *Miwi2* mutant testes to assess the integrity of the mutant soma. Recipient animals reconstituted complete spermatogenesis in a subset of tubules, with successful completion of both meiotic divisions and production of mature sperm (Figure 6E, lower). These spermatogenic tubules existed side by side with noncolonized tubules that displayed the characteristic *Miwi2* mutant phenotype (Figure 6E, upper). Although our conclusions must be tempered by the remote possibility that the mutant soma could harbor a level of *Miwi2* that escapes detection by RT-PCR, these studies strongly suggest that *Miwi2* mutant soma can successfully support germ cells and lead to the conclusion that wild-type levels of *Miwi2* expression in the germ cells themselves is necessary and sufficient to support meiosis and spermiogenesis.

A Role for MIWI2 in Transposon Control

Two lines of circumstantial evidence point to a potential role for mammalian Piwi proteins in transposon control. First, in *Drosophila*, Piwi proteins have a demonstrated role in the control of transposons (Aravin et al., 2001, 2004; Kalmykova et al., 2005; Saito et al., 2006; Sarot

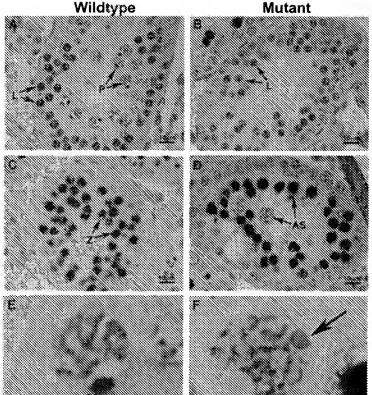

Figure 4. Mutant Spermatocytes Show Abnormalities in a Marker for Double-Strand Breaks

(A–F) Phosphorylated histone H2AX (γ-H2AX) staining (brown) of (A, C, and E) wild-type and (B, D, and F) mutant seminiferous tubules of 14-day-old animals. Tubules containing (A and B) leptotene, (C) zygotene, and (A and E) pachytene cells are shown. Leptotene spermatocytes are indicated by L, zygotene by Z, pachytene by P, and abnormal spermatocytes by AS. The sex body in the wild-type pachytene spermatocyte is indicated with an arrow in (E). A similar structure in the mutant that fails to stain is indicated with an arrow in (F).

et al., 2004; Savitsky et al., 2006; Vagin et al., 2004, 2006). Transposon activation results in both germline and embryonic defects that result in female sterility through a phenomenon called hybrid dysgenesis. This is characterized by a depletion of germline stem cells, abnormal oogenesis, and defects in oocyte organization. Second, a link between the inappropriate expression of certain repetitive elements and meiotic arrest has previously been demonstrated in mammals. In particular, animals bearing mutations in a catalytically defective member of the DNA

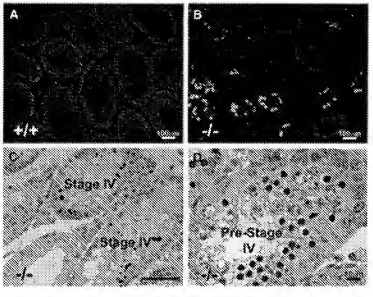

Figure 5. Mutant Testes Exhibit High Levels of Apoptotic Cell Death

(A and B) TUNEL assay on 3-month-old (A) wild-type and (B) mutant animals. Apoptotic cells are labeled with FITC (green), and DAPI stained nuclei are blue.

(C and D) Colorimetric TUNEL assay with positive nuclei staining brown. (C) 3 month old animal with apoptosis in epithelial stage IV, and (D) a 15 dpp tubule that has not yet reached stage IV.

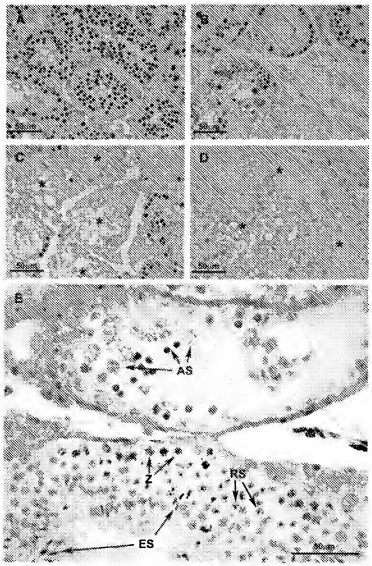

Figure 6. *Miwi2* Mutant Testes Progressively Lose Germ Cells

(A–D) Germ cells are stained with anti–germ cell nuclear antigen (GCNA) (brown) in (A) 15-day-, (B) 3-month-, (C) 6-month-, and (D) 20-month-old animals. Sertoli-cell-only tubules are indicated with an asterisk.

(E) A *Miwi2* mutant testis that has been transplanted with wild-type germ cells is shown. A noncolonized tubule displaying the *Miwi2* mutant phenotype is at the top. Abnormal mutant spermatocytes are indicated by AS. The lower tubule has been colonized by wild-type germ cells. Normal spermatocytes and spermatids are indicated. Zygotene nuclei are indicated by Z, haploid round spermatids are indicated by RS, and elongating spermatids are indicated by ES.

methyltransferase family, DNMT3L, fail to methylate transposons in the male germline, resulting in abnormal and abundant expression from several transposon families (Bourc'his and Bestor, 2004; Hata et al., 2006; Webster et al., 2005). This phenomenon is correlated with a meiotic arrest prior to pachytene as well as germ cell loss. We therefore considered that the germ cell loss and prevalent apoptosis that we observe in *Miwi2* mutants might correlate with transposon activation.

To investigate whether *Miwi2* mutation affected expression from normally silent transposons, we used in situ hybridization. When using this method, long interspersed elements (LINEs) are not detectable in adult wild-type testes (Figure 7A). However, in *Miwi2* mutants, a strong signal can be seen with probes that detect sense-oriented LINE-1 transcripts (Figure 7B). Similar approaches were also used to monitor expression of intracisternal A particle (IAP) elements that belong to the most active class of LTR retrotransposons in the mouse. Sense strand IAP transcripts were undetectable by in situ hybridization in wild-type animals, while they were readily detectible in *Miwi2* mutants (Figures 7C and 7D). Elevated levels of transcripts were detected exclusively in germ lineages, with no apparent activation in Sertoli or interstitial cells of the testes.

Results from in situ analyses were supported and extended by quantitative RT-PCR (Figure 7E). A 7- to 12-fold Increase in LINE-1 expression was detected in the mutants relative to heterozygous animals when primers directed to the 5′UTR and ORF2 were used. Similar results were obtained with strand-specific RT-PCR measuring only sense-orientation LINE-1 transcripts (not shown). IAP elements were activated more modestly. Elevated expression of these elements was detected only in the testes, and not in the kidneys, of mutant animals

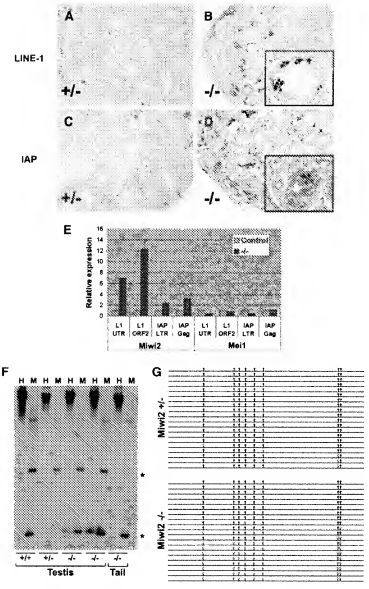

Figure 7. *Miwi2* Mutants Derepress and Demethylate Transposable Elements

(A–D) In situ hybridization of testes of the indicated genotypes with probes recognizing the sense strands of (A and B) LINE-1 and (C and D) IAP elements.

(E) Quantitative RT-PCR analysis of transposable elements in 14-day-old animals. Data are represented as mean ± SEM.

(F) DNA isolated from the tail or testes of wild-type, heterozygous, and *Miwi2* mutant animals was digested with either methylation-insensitive (MspI, M) or methylation-sensitive (HpaII, H) restriction enzymes. Southern blot analysis of these DNAs was conducted, and membranes were probed with a fragment of the LINE-1 5′UTR. Asterisks indicate the expected band sizes. The probe recognizes four bands of 156 bp generated by HpaII sites in the 5′UTR, and a band of 1206 bp that is generated by one HpaII site in the 5′UTR and one site in the coding sequence.

(G) Lollipop representation of the sequences obtained after bisulfite treatment of *Miwi2* +/− and −/− testis DNA. The first 150 bp of a specific L1 element were selectively amplified and analyzed for the presence of methylated CpGs. Methylated and unmethylated CpGs are represented as filled and empty lollipops, respectively. Out of 75 sequences obtained for each genotype, 20 randomly chosen sequences are shown. Information on the complete set can be found in Figure S3.

(data not shown). To ensure that the observed effects were not a secondary consequence of meiotic arrest, we analyzed testes from *meiosis defective-1* (*Mei1*) mutant animals, which display a meiotic arrest phenotype similar to *Miwi2* mutants, and failed to observe increased transposon expression (Figure 7E).

Transposable elements are thought to be maintained in a silent state by DNA methylation and packaging into heterochromatin. We investigated the methyation status of LINE-1 in the *Miwi2* mutants by Southern blot analysis after digestion with a methylation-sensitive enzyme, HpaII, and found that LINE-1 elements become demethylated in *Miwi2* mutants as compared to wild-type and heterozygous animals (Figure 7F). Demethylation was detected specifically in DNA prepared from the testes and not from the tail. Thus, compromising *Miwi2* can affect the methylation of repetitive elements specifically in the germline. For comparison, we assayed LINE-1 methylation in testes from several mutants that show a meiotic arrest similar to *Miwi2* mutants (Figure S2). None of these mutant animals show LINE-1 demethylation.

We used bisulfite sequencing to examine methylation of the first 150 bp of the 5′UTR of a specific copy of L1Md-A2. In heterozygous animals, this region is almost completely methylated, with 95% of all CpGs modified (Figure 7G). In the mutant, only 60% of CpGs are methylated overall, with two distinct populations of PCR products being apparent. These are represented at the extremes by 34% of the clones that are completely unmethylated, and 46% that retain full methylation (Figure 7G;

Figure S3). Based on our Southern blot and quantitative RT-PCR analyses that show normal methylation and transposon repression in somatic tissues, we suggest that these two populations are likely derived from germ cells (unmethylated) and somatic cells (methylated).

DISCUSSION

Successful expansion by selfish genetic elements can only occur if increased copy numbers can be transmitted to the next generation. Consistent with this notion, LINE and IAP elements are known to be active almost exclusively in the germline (Branciforte and Martin, 1994; Dupressoir and Heidmann, 1996). Full-length sense strand LINE-1 transcripts, and the ORF1 protein that they encode, have been detected in leptotene and zygotene spermatocytes in pubertal mouse testes (Branciforte and Martin, 1994). In the adult male, truncated transcripts and ORF1 protein are present in somatic cells and haploid germ cells (Branciforte and Martin, 1994; Trelogan and Martin, 1995). ORF1 protein is also present in oocytes and steroidogenic cells in the female germline (Branciforte and Martin, 1994; Trelogan and Martin, 1995). Considering the deleterious and cumulative effects of unregulated repetitive element expansion, there should be tremendous evolutionary pressure to evolve effective transposon control strategies in the germline. Our data indicate that mammalian Piwi proteins form at least part of such a defense mechanism.

In *Drosophila*, Piwi proteins are reported to have both cell autonomous and nonautonomous roles in maintaining the integrity of the germline (Cox et al., 2000). In particular, *piwi* mutants lose germ cells as a result of functions for this protein in the germ cells themselves and in maintaining the integrity of the germline stem cell niche. In mammals, *Miwi* and *Mili* mutants arrest spermatogenesis at different stages, but neither is reported to lose germ cells, as might be expected if, like PIWI, either protein had a role in stem cell maintenance. Here, we show that disruption of *Miwi2* creates two distinct phenotypes in the male germline of mice. First, *Miwi2* mutant germ cells that enter prophase of meiosis I arrest prior to the pachytene stage. Second, *Miwi2* mutants progressively lose germ cells and accumulate tubules that contain only somatic Sertoli cells. The latter observation suggests that MIWI2 may conserve some of the stem cell maintenance functions played by PIWI in *Drosophila*. It is presently unclear whether the requirement for Piwi proteins in stem cell maintenance in flies is due to their role in regulating gene expression, or whether the phenotypes of Piwi-family mutations can be solely explained by loss of transposon control.

Accumulating data have suggested that *Drosophila* Piwi proteins play a prominent and essential role in transposon control (Aravin et al., 2001, 2004; Kalmykova et al., 2005; Sarot et al., 2004; Savitsky et al., 2006; Vagin et al., 2004). One consequence of disrupting transposon suppression in flies is the appearance of DNA damage, as evidenced by the accumulation of phosphorylated histone H2AX (Belgnaoui et al., 2006; Gasior et al., 2006) A key role for DNA-damage pathways in the ultimate output of Piwi-family mutations, production of defective oocytes, is indicated by the fact that mutation of key DNA-damage sensing pathways can at least partially suppress the effects of transposon activation (Klattenhoff et al., 2007). Our results point to a previously unsuspected role for mammalian Piwi proteins in the control of transposons in the male germline. As in flies, *Miwi2* mutations also result in accumulation of DNA damage, as indicated by γ-H2AX accumulation. The relationship between the molecular phenotypes of Piwi-family mutations in flies and mice, particularly whether activation of DNA-damage response pathways plays a role in the meiotic defects observed in *Miwi2* mutants, remains to be determined.

Drosophila Piwi proteins interact with small RNAs of ~24–26 nucleotides in length (Aravin et al., 2001; Saito et al., 2006; Vagin et al., 2006). These are highly enriched for sequences that target repetitive elements and are therefore called rasiRNAs (repeat-associated siRNAs) (Aravin et al., 2003; Saito et al., 2006). In contrast, mammalian Piwi-family proteins, MIWI and MILI, bind to an ~26–30 nucleotide class of small RNAs known as piRNAs (Piwi-interacting RNAs) (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006; Watanabe et al., 2006). A large proportion of piRNAs are only complimentary to the loci from which they came, leading to the hypothesis that the piRNA loci themselves must be the targets of MILI and MIWI RNPs. Results presented here point to a role for piRNAs in transposon control in mammals similar to those that have been demonstrated for rasiRNAs in *Drosophila*.

Unexpectedly, we have found that the rasiRNA system in flies shows many characteristics in common with the piRNA system in mammals (Brennecke et al., 2007). Piwi-interacting RNAs in *Drosophila* are derived from discrete genomic loci. At least some of these loci show the profound strand asymmetry that characterizes mammalian piRNA loci. These observations begin to unify Piwi protein functions in disparate organisms. However, future work will be required to understand how the meiotic piRNA loci, which are depleted of repeats, relate functionally to the piRNA loci in flies that act as master controllers of transposon activity.

Silencing of mammalian transposons depends on their methylation status (Bourc'his and Bestor, 2004). Genomes of primordial germ cells undergo demethylation followed by de novo remethylation in prospermatogonia, a nondividing cell type that exists only in the perinatal period. How the patterns of methylation are determined in developing germ cells is not understood. In *Arabidopsis*, it is well established that the RNAi machinery can use small RNAs to direct genomic methylation, though the precise biochemical mechanism underlying these events remains unclear (Matzke and Birchler, 2005). In plants, ARGONAUTE4, a member of the Argonaute rather than the Piwi subfamily, binds to 24 nt, small RNAs and mainly directs asymmetric cytosine methylation (CpNpG and CpHpH). However, such asymmetric methylation is rare or absent in mammalian genomes. Here, we provide

evidence that loss of MIWI2 function affects the methylation status of LINE-1 elements. MIWI2 complexes, which we presume are directed to their targets by associated piRNAs, might help to establish genomic methylation patterns on repetitive elements during germ cell development. It is also possible that removal of MIWI2 interferes with the maintenance of genomic methylation patterns that normally occurs in dividing spermatogonia. A detailed analysis of patterns of *Miwi2* expression and identification of piRNAs that interact with MIWI2 during germ cell development will be needed to distinguish roles for this protein complex in de novo versus maintenance methylation.

EXPERIMENTAL PROCEDURES

Gene Targeting and Mice

The *Miwi2* targeting construct was obtained by screening of the lambda phage 3' HPRT library described by Zheng et al. (1999) that is now the basis of the MICER system (Adams et al., 2004). The resultant targeting construct, containing exons 9–12 of *Miwi2*, was electroporated into AB2.2 mouse embryonic stem (ES) cells. Targeted clones were injected into C57BL/6 blastocysts to generate eight high percentage chimeras, four of which were able to pass the allele through the germline. Results presented herein were obtained from mice with a mixed 129/B6 background. In general, younger animals were backcrossed to B6 4–6 generations, and older animals were backcrossed less. Mouse genotyping was performed by Southern blot analysis after digestion of genomic DNA with AccI, The 332 bp probe was amplified from genomic DNA with primers described in Table S1.

Histology

Testes were collected and fixed in Bouin's fixative at 4°C overnight, then dehydrated to 70% ethanol. After embedding in paraffin, 8 μm sections were made by using a microtome. For routine histology, sections were stained with hematoxylin and eosin. For routine histology and subsequent staining, at least three animals of each age and genotype were examined.

Immunohistochemistry

Slides were rehydrated and treated with 3% hydrogen peroxide for 10 min. Blocking was carried out in 5% goat serum, 1% BSA in PBS for 10 min. Slides were incubated overnight at 4°C with primary antibody as follows. Antibody to γ-H2AX (Upstate) was used at 1:150 in 1% BSA in PBS. GCNA (a gift of G. Enders) was used neat. Detection was performed by using the Vector ABC kit according to the manufacturer's directions, except 2 μl each of solutions A and B were used per milliliter of PBS. Slides were counterstained with Mayer hematoxylin, mounted with Histomount mounting media, and coverslipped.

For immunocytological analysis of synaptonemal complex formation, surface spreading of spermatocytes was performed as described by Matsuda et al. (1992). Spreads were hybridized with goat anti-Scp3 (gift of T. Ashley) at 1:400 dilution. Approximately 200 nuclei from each of three animals were counted, for a total of 600 nuclei of each genotype. Spreads were conducted on animals at 16 dpp.

TUNEL Assay

Slides containing Bouin's-fixed testes sections were rehydrated and microwaved for 5 min in 10 mM Citrate buffer (pH 6.0). After incubation in 3% hydrogen peroxide, slides were incubated with 0.3 U/microliter deoxynucleotidal terminal transferase (Amersham) and 6.66 μM biotin-16-dUTP (Roche) for 1 hr at 37°C. After washing in 300 mM NaCl, 30 mM NaCitrate in MilliQ water for 15 min at room temperature, slides were blocked in 2% BSA in PBS for 10 min. Slides were incubated in a 1:20 dilution of ExtrAvidine peroxidase (Sigma) in 1% BSA in PBS for 30 min at 37°C. Detection was achieved by using diaminobenzidine. Slides were counterstained with Mayer hematoxylin, dehydrated, and mounted. Fluorescent TUNEL assay was conducted by using the Roche In Situ Cell Death Detection kit according to the manufacturer's instructions.

Germ Cell Transplants

Transplants were carried out as described by Buaas et al. (2004). Donor cells were harvested from the transgenic mouse line C57BL/6.129-*Tgfi(Rosa26)26S* (Jackson Laboratory). Donor cells were transplanted into testes of *Miwi2* mutant mice that were already somewhat germ cell depleted due to the mutation, or into *W/W*^{v} mice that have no endogenous spermatogenesis as a control (Jackson Laboratory, WBB6F1/J-*Kit*^{W}/*Kit*^{W_v}). Recipient testes were analyzed with standard histological methods to identify areas of colonization by donor cells. One out of 10 *Miwi2* mutant recipients and 2 out of 5 *W/W*^{v} were successfully colonized.

RT-PCR and QPCR

Total RNA was extracted from mouse tissues by using Trizol according to the manufacturer's recommendations. cDNA was synthesized by using Superscript III Reverse Transcriptase (Invitrogen) on RNA primed with random hexamers. QPCR was carried out by using Sybr Green PCR Master Mix (Applied Biosystems) on a Biorad Chromo 4 Real Time system. Two animals of each genotype were examined, with the exception of *Mer1*, for which we had only one specimen. Assays were done in triplicate. *Miwi2* animals were 14 days old, and Mei1 animals were 21 days old. Primers Miwi2-F and Miwi2-R are downstream of the duplicated exons and cannot distinguish between wild-type and mutant transcript. Primers Miwi2-exon7F and Miwi2-exon14R flank the duplicated exons in the mutant transcript and therefore assay for only the wild-type transcript. The wild-type transcript produces a band of 1006 bp, while the mutant would yield a larger product due to the duplication of exons 9–12. Primers are listed in Table S1.

In Situ Hybridization

In situ hybridization was done as described by Bourc'his and Bestor (2004). The 5'LTR IAP probe was as described by Walsh et al. (1998), and the LINE-1 5'UTR probe is complementary to a type A LINE-1 element (GenBank accession number: M13002, nucleotides 515–1,628) (Bourc'his and Bestor, 2004).

Methylation Southern Blot Analysis

Southern blot analysis to assay for methylation was done as described by Bourc'his and Bestor (2004). The same LINE-1 5'UTR probe was used as for in situ hybridization, except a gel-purified fragment was random prime labeled by using the Rediprime II kit (Amersham). DNA from testis and tail were digested with the methylation-sensitive enzyme HpaII and its methylation-insensitive isoschizomer, MspI.

Bisulfite DNA Sequencing

DNA from *Miwi2* +/− and −/− testes was bisulfite treated and purified by using the EZ DNA Methylation Gold kit (Zymo Research). Primers MethylL1-F and MethylL1-R were designed to specifically amplify one occurrence of L1Md-A2 located on chromosome X. The PCR products were then gel purified, TOPO cloned (Invitrogen), sequenced, and analyzed by using BiQ-Analyzer (Bock et al., 2005). Primers and the sequence of the amplified region are given in Table S1.

Supplemental Data

Supplemental Data include analysis of *Miwi2* expression, transposon demethylation controls, the entire bisulfite DNA-sequencing data set, and primer sequences and are available at http://www.developmentalcell.com/cgi/content/full/12/4/503/DC1/.

ACKNOWLEDGMENTS

We thank Takeshi Soda and Pedro Aponte (Utrecht University) for instruction in germ cell transplantation. We also thank Terry Ashley and Adelle Hack (Yale) for instruction on spermatocyte spreads,

antibodies, and helpful discussions. We thank Peter de Boer (University of Wageningen) for helpful discussion, Stephen Hearn (Cold Spring Harbor Laboratory [CSHL]) for microscopy assistance and helpful discussions, and George Enders (University of Kansas) for GCNA antibody. We thank Ignasi Roig and Scott Keeney (Memorial Sloan Kettering Cancer Center) for tissues from Atm and Dmc1 mice, and Xin Li and John Schimenti (Cornell) for tissue from Mei1 mice. We thank Lisa Bianco, Jodi Coblentz, Lea Manzella, and Gula Nourjanova (CSHL) for animal assistance and histology. We thank Mike Griswold and Lizhong Yang (Washington State University) for access to unpublished data. We thank members of the Hannon laboratory for comments on the manuscript. A.G. is a Florence Gould Fellow of the Watson School of Biological Sciences. G.J.H. is an investigator of the Howard Hughes Medical Institute. This work was supported by grants from the National Institutes of Health (G.J.H).

Received: November 8, 2006
Revised: February 28, 2007
Accepted: March 1, 2007
Published online: March 28, 2007

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